Interactions of amiloride and small monovalent cations with the epithelial sodium channel

Inferences about the nature of the channel pore

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ABSTRACT The voltage dependence of amiloride-induced inhibition of current flow through apical membrane sodium channels in toad urinary bladder was studied at different ionic conditions. The "inert" salt *N*-methyl-D-glucamine HCl (NMDG HCl) affected neither the apparent inhibition constant (K_i) for the amiloride-induced current inhibition nor the apparent fraction of the transmembrane voltage that falls between the mucosal solution and the amiloride-binding site (δ). When $NMDG^+$ was replaced with Na^+, K_i increased, reflecting amiloride-Na^+ competition, whereas δ was unchanged. Similar results were obtained with another permeant cation, Li^+. When $NMDG^+$ was replaced by K^+, an impermeant but channel-blocking cation, K_i increased whereas δ decreased. Similar results were obtained using another impermeant, channel-blocking cation guanidinium. The results are interpreted on the premise that Na^+ and K^+ compete with amiloride by binding to cation binding sites within the channel lumen such that ion occupancy of these sites vary with voltage. Occupancy by K^+, which cannot traverse the channel, will increase as the mucosal solution becomes positive, relative to the serosal solution. Occupancy by Na^+, which can traverse the channel, is comparatively voltage independent. Ion movement through the channels was simulated using discrete-state kinetic models. Two types of models could describe the shape of the current-voltage relationship and the voltage dependence of the amiloride-induced channel block. One model had a single ion-binding site with a broad energy barrier at the inner (cytoplasmic) side of the site. The other model had two binding sites separated from each other and from the aqueous solutions by sharp energy barriers.

INTRODUCTION

Amiloride is a potent, reversible inhibitor of ion movement through epithelial sodium channels (Benos, 1982). In the toad urinary bladder, the amiloride-induced current inhibition is voltage dependent: transmembrane voltages that would tend to drive the positively charged amiloride from the mucosal solution into the membrane (channel) increase its apparent affinity for its binding site (Palmer, 1984*a*, 1985 Warncke and Lindemann, 1985; Hamilton and Eaton, 1985 Marunaka and Eaton, 1988). One possible explanation for this observation is that amiloride can enter the channel lumen, thereby forming a molecular plug that obstructs the flow of permeant ions. In addition, permeant cations, such as Na^+, and poorly permeant cations, such as K^+, interact competitively with amiloride in this tissue (Palmer, 1985; Warncke and Lindemann, 1985). This suggests that amiloride binds within the channel's pore, thereby inhibiting ion movement, and that the competition by Na^+ or K^+ is exerted when these ions bind in the pore. In the simplest scheme, Na^+ interacts with the amiloride binding site as an early step in the permeation process. In this paper we investigate this possibility further by examining the voltage dependence of the cation-amiloride-channel interactions. Discrete kinetic models with two ion-binding sites and three sharp kinetic barriers or with a single binding site and one sharp and one broad energy barrier are compatible with the experimental results.

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METHODS

Experiments were performed using the urinary bladder of the toad, *Bufo marinus* (National Reagents, Bridgeport, CT). The bladders were bathed on their serosal sides with a high K^+ solution to depolarize the basal-lateral membrane and reduce its electrical resistance. The rationale for this approach has been discussed elsewhere (Palmer et al., 1980; Palmer, 1984*b*).

The serosal bathing solution contained (in mM): KCl, 85; sucrose, 50; $CaCl_2$, 1; $MgCl_2$, 0.5; glucose, 5; and Hepes, 5; buffered to pH 7.5 with NaOH. The basic mucosal solution contained NaCl, 115; $CaCl_2$, 1; $MgCl_2$, 0.5; and MES, 5; buffered to pH 6.0 with NaOH. In some experiments the [NaCl] in the mucosal solution was reduced to 30 mM and was either not replaced, resulting in a hypotonic mucosal medium, or was replaced by an equimolar amount of KCl, LiCl, guanidine HCl, or *n*-methyl-*d*-glucamine HCl (NMDG HCl). Amiloride was a gift of Merck, Sharp and Dohme (West Point, PA). It was added to the mucosal solution at either submaximal concentrations (0.1 – 0.4 μM) or maximal concentrations (10 μM). The tissues were voltage-clamped, and all currents are reported as the amiloride-inhibitable current (I_{Na}). It was obtained by subtracting currents, at the same voltage and ionic conditions, but in the presence of 10 μM amiloride. The mounting of the

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tissue and the electrical recording techniques were described previously (Palmer, 1984*a*, 1985).

The basic protocol was to allow the bladder to reach steady-state conditions at various mucosal ionic compositions in the absence of amiloride. A series of solution changes was then performed in which the concentration of amiloride was increased in steps (at submaximal doses), followed by a maximal dose. After a solution change, the new steady-state short-circuit current was generally attained within 1 min. The amiloride was then washed away, a new mucosal solution with a different ionic composition was introduced, and the process was repeated. Up to four different ionic solutions were tested on one hemibladder. The order of the solution changes was varied to reduce the possibility of systematic variations.

CALCULATIONS

The apparent inhibition constant for amiloride (K_I) was computed from the fractional change in the transepithelial current, I_{Na}, determined under short-circuit conditions, $I_{Na}(A) = I_{Na}(0)/(1 + [A]/K_I)$, or

$$K_I = [A] \cdot I_{Na}(A)/[I_{Na}(0) - I_{Na}(A)], \quad (1)$$

where [A] is the amiloride concentration, $I_{Na}(0)$ is the current in the absence of amiloride, and $I_{Na}(A)$ the current in the presence of amiloride. The apparent competition constant for K^+'s competition with amiloride (K_K) was estimated from $K_I(K) = K_I(0)(1 + [K^+]/K_K)$, or

$$K_K = K_I(0)\,[K^+]/[K_I(K) - K_I(0)], \quad (2)$$

where $K_I(K)$ denotes the inhibition constant for the amiloride-induced current inhibition in the presence of K^+, and $[K^+]$ is the mucosal K^+ concentration ($K_I[0]$ was estimated from results obtained with $NMDG^+$ as the major mucosal cation). It was shown previously that the dose-response curve for amiloride can be described by Eq. 1 and that K^+, as well as Na^+, exert competitive effects on the amiloride-induced current inhibition (Palmer, 1984*a*).

The voltage dependence of the amiloride-induced current inhibition was assessed using the Woodhull model for channel block (Woodhull, 1973):

$$I_{Na}(A,U) = I_{Na}(0,U)/\{1 + [A]/K_I(U)\}$$
$$= I_{Na}(0,U)/(1 + [A]/K_I(0) \cdot \exp\{\delta \cdot U\}), \quad (3)$$

where $U = (RT/F)\,V_T$, R is the gas constant, T is the temperature in Kelvin, F is Faraday's constant, and V_T is the transepithelial voltage (which is assumed to be equal to of the apical transmembrane voltage when the basolateral membrane is depolarized). $K_I(0) \cdot \exp\{\delta \cdot U\}$ is the (voltage-dependent) apparent inhibition constant for amiloride, $K_I(U)$, where δ is the fraction of the applied voltage that appears to be sensed at the amiloride binding site. δ is estimated from plots of $ln\{[A]/K_I(U)\}$ as a function of U, i.e., as

$$ln\,[I_{Na}(0,U)/I_{Na}(A,U) - 1] = ln\,([A]/K_I) + \delta \cdot U. \quad (4)$$

Details and justification of this procedure have been described previously (Palmer, 1984*a*; cf Woodhull, 1973).

RESULTS

In the experiments described here, we examined the specific effects of alkali metal cations Na^+ and K^+ on the interaction of amiloride with the apical sodium channels in the toad urinary bladder. To facilitate interpretation of the results, it was necessary to have an "inert" cation that has little effect on Na^+ transport per se or on its inhibition by amiloride. Cations with diameters larger than about 5 Å, e.g., $NMDG^+$, have no apparent effect on Na^+ movement through the apical channels (Palmer, 1985). $NMDG^+$ likewise has little effect on the amiloride-induced current inhibition. Neither the apparent inhibition constant for amiloride, K_I (see Eq. 1), nor the voltage dependence of the current inhibition, δ (see Eq. 4), was affected by adding NMDGCl to a hypotonic mucosal solution at a constant mucosal $[Na^+]$ (see Table 1). This implies that $NMDG^+$ does not interact competitively with amiloride (directly or indirectly by altering Na^+-amiloride interactions). $NMDG^+$ was therefore considered to be inert for the purposes of the experiments reported in the rest of this paper.

The basic experiment is illustrated in Fig. 1, where we for three different mucosal cations ($NMDG^+$, Na^+, and K^+) show the current responses to sudden changes in transepithelial voltage, from 0 to +200 mV (mucosa positive), in the presence and absence of amiloride. These transients are typical of those seen at all positive voltages. In the absence of amiloride, the currents are fairly constant, tending to increase slightly with time. In the presence of a submaximal amiloride concentration, there is a downward relaxation that results from the voltage and time dependence of the amiloride-induced current inhibition. The time constant of the exponential decay reflects the kinetics of the block (Palmer, 1985). The amplitude of this transient is much reduced in the presence of a high mucosal $[K^+]$ (Fig. 1, *bottom*). This implies that the amiloride-induced current inhibition is less voltage dependent when K^+ is the major mucosal cation than when Na^+ or $NMDG^+$ are the major cations.

This key experimental result was pursued further as shown in Fig. 2, where the apparent affinity of amiloride for its binding site is plotted as a function of V_T. Ratios of I_{Na} in the absence and presence of amiloride were computed from the currents measured in the steady state, at

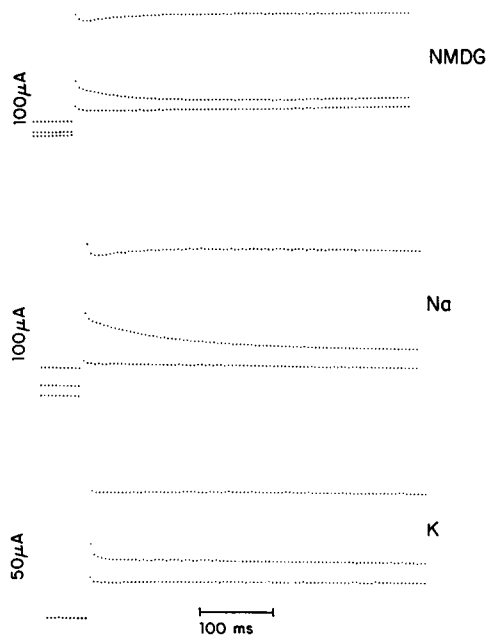

FIGURE 1 Current transients in response to a change in voltage with Na^+, $NMDG^+$, or K^+ as the major mucosal cation. A single hemibladder was equilibrated with 30 mM NaCl plus 85 mM KCl on the mucosal side (*bottom*). The voltage was held at zero and stepped to various positive voltages between +20 and +200 mV (mucosal side positive to serosal) for 450 ms. Each pulse was followed by a 450-ms period at 0 mV. A submaximal dose of amiloride (0.4 μM) was then added to the mucosal solution and the same voltage jumps were performed. Finally, a maximal dose (10 μM) of amiloride was added and the procedure was again repeated. In this figure only current transients in response to the 200-mV pulse are illustrated. Top trace, no amiloride; middle trace, 0.4 μM amiloride; bottom trace, 10 μM amiloride. The mucosal solution was then changed to one containing 85 mM LiCl (not shown), 85 mM NMDG HCl (*top*), and finally 115 mM NaCl (*middle*). The same protocol was followed in each case. The middle traces in each panel show the characteristic slow relaxation of current in the presence of a submaximal dose of amiloride, reflecting the voltage-dependent amiloride-induced current inhibition.

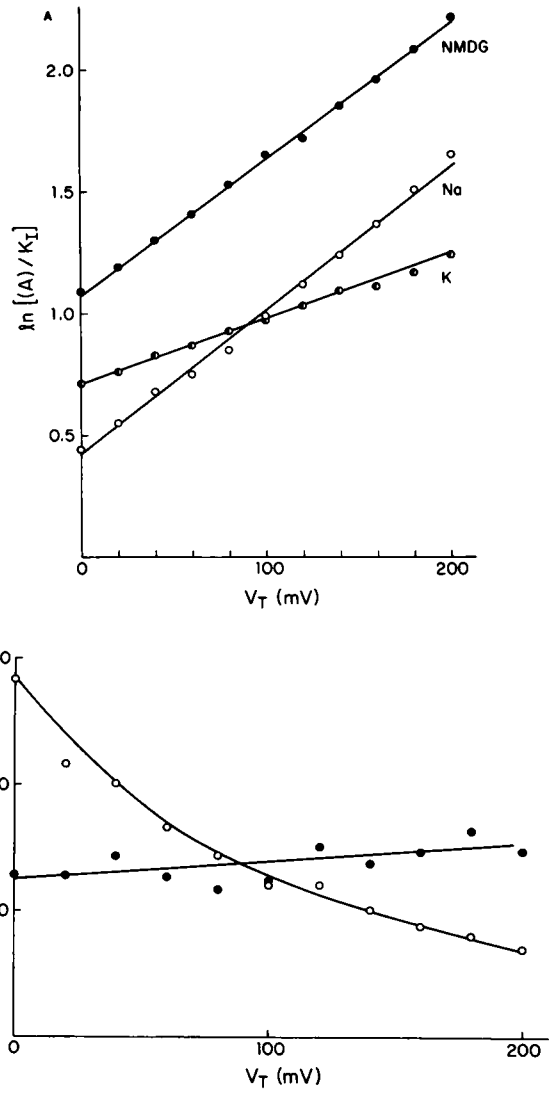

FIGURE 2 Voltage dependence of the amiloride-induced current inhibition in the presence of mucosal $NMDG^+$, Na^+, or K^+. (*A*) The steady-state currents from the experiment in Fig. 1 were corrected for amiloride-insensitive currents and plotted as a function of voltage according to Eq. 4. The straight lines were fitted to the results using least squares linear regression. The slopes of the plots give the apparent voltage dependence of the block (δ) the intercepts give the apparent inhibition constant (K_I) at V_T = O. voltage. (*B*) The apparent competition constants for Na^+ and K^+ with respect to amiloride block are plotted as functions of voltage.

the end of the 450-ms pulses shown in Fig. 1. The increase in apparent affinity with voltage is, again, a reflection of the voltage dependence of the current inhibition (Palmer, 1984*a*). The curves with $NMDG^+$ and Na^+ as the principal mucosal cations are parallel within experimental error. The downward displacement of the curve in the presence of Na^+ indicates that Na^+ and amiloride interact competitively; Na^+ reduces the amiloride's affinity for the binding site. That the displacement is nearly the same at all voltages implies that the competition by Na^+ does not depend on the applied voltage. In contrast, in the presence of K^+, the competition with amiloride has a different voltage dependence than in the other experiments. At $V_T = 0$, K^+ exerts a weaker competitive effect than Na^+, at $V_T = 200$ mV, K^+ exerts a stronger competitive effect than Na^+. This is illustrated further in Fig. 2 *B*, where the apparent competition constants for Na^+ and K^+ are plotted as functions of V_T. Results of a number of experiments with Na^+, K^+, and $NMDG^+$ are summarized in Table 1. An increase in $[Na^+]$ increased K_I but not δ, whereas addition of K^+ increased K_I but reduced δ.

TABLE 1 Effect of mucosal cations on the amiloride-induced inhibition of current flow through epithelial sodium channels

Cation	K_I	δ	No. of experiments
	μM		
—	0.20 ± 0.02	0.11 ± 0.01	12
$NMDG^+$	0.20 ± 0.02	0.11 ± 0.01	12
$NMDG^+$	0.11 ± 0.01	0.14 ± 0.01	11
Na^+	0.31 ± 0.03	0.16 ± 0.01	11
K^+	0.23 ± 0.02	0.068 ± 0.006	11
$NMDG^+$	0.13 ± 0.01	0.15 ± 0.01	6
Li^+	0.21 ± 0.01	0.14 ± 0.01	6
$NMDG^+$	0.09 ± 0.01	0.14 ± 0.01	5
Guanidinium	0.33 ± 0.04	0.07 ± 0.01	5

K_I was computed from the current ratio at $V_T = 0$ using Eq. 1. δ was computed using Eq. 4. Paired experiments, in which results were obtained with $NMDG^+$ and a test ion using the same hemibladders are grouped together. The results are given as means ± SEM.

These results are consistent with the idea that K^+ blocks apical sodium channels with a voltage dependence that is similar to that of amiloride (Palmer, 1984*a*). As the voltage is increased, both amiloride and K^+ are driven into the channel and the inhibition constants for both amiloride and for K^+ are decreased. This means that channel occupancy by K^+ increases with increasing voltages, and the increase in amiloride affinity is partially offset by the increased competitive strength of the K^+.

If this is the explanation for the K^+ effect on δ, then why is the competitive effect of Na^+ not enhanced by voltage? One possibility is that Na^+ and K^+ act at different sites, that for K^+ being inside the channel and that for Na^+ being outside the channel and thus insensitive to changes in the transmembrane voltage. Another explanation, which we find simpler and more attractive, is that, because Na^+ is permeant, an increase in voltage will tend to drive Na^+ into the channels to compete with amiloride but it will also tend to pull Na^+ out of the channels into the cytoplasm. This reduces the voltage dependence of Na^+ occupancy and thus the voltage dependence of Na^+'s competitive effect (Woodhull, 1973). In fact, if there is no voltage dependence of the Na^+ occupancy, the competitive strength of Na^+ will be voltage independent.

To test this hypothesis, the effects of another permeant cation, Li^+ (Palmer, 1982*a*), and another impermeant cation that shows voltage dependent block, guanidinium (Palmer, 1985), were tested. As summarized in Table 1, Li^+, like Na^+, increased K_I for amiloride but did not detectably alter δ. Guanidinium, like K^+, increased K_I and reduced δ. Thus, qualitatively, the hypothesis is supported.

A more quantitative assessment of the K^+ effect was done by assuming that amiloride binds to a site that senses a fraction δ_A of the electric field, whereas K^+ binds to a site that senses a fraction δ_K of the field. It was further assumed that occupancy by amiloride and K^+ is mutually exclusive. Under these circumstances, the observed δ for the amiloride-induced block is given by:

$$\delta = \delta_A - \delta_K/(1 + (K_K/[K^+]) \cdot \exp\{-\delta_K \cdot U\}). \quad (5)$$

Using the estimate for δ near $U = 0$ (0.068 ± 0.006) and K_K values calculated using Eq. 2 (110± 15 mM, which means that $K_K/[K^+] \simeq 1.3$), the mean value of δ_K was 0.14 ± 0.02, which is similar to the δ_A estimate in Table 1. This suggests that amiloride and K^+ interact by binding at a single site or at two sites that sense similar voltage. (According to Eq. 5, δ should decrease as U increases. In practice plots such as that in Fig. 2 *A* were approximately linear, implying that δ was fairly constant over the voltage range used in these experiments. We do not believe this is a problem, however, because $1/\{1 + (K_K/[K^+]) \cdot \exp(-\delta_K \cdot U)\}$ increases only little as a function of voltage: from 0.45 to 0 mV, to 0.52 at 100 mV, and 0.58 at 200 mV. δ should thus have a quite weak voltage dependence.)

The purpose of these experiments was to obtain additional information about the interactions between amiloride, monovalent cations, and the epithelial sodium channel. To this end we also determined the shape of the current-voltage relationship (Fig. 3). At positive V_T, the slope conductance is somewhat less that that predicted from the constant-field equation (Palmer, 1984*a*). This prediction is denoted by the stippled curve. The solid curve that describe the results was obtained using the two-site model described below.

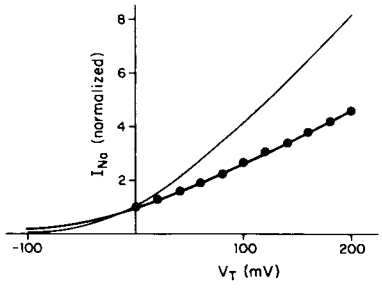

FIGURE 3 Steady-state current relationship with 115 M NaCl in the mucosal solution. The results are from the experiment in Fig. 1. The solid line represents the best fit of the two-site-three-barrier model (see Scheme 2 and Eqs. 9–13). The parameters are: $\kappa^{IO}_{OO}/\kappa^{OO}_{OI} = \kappa^{OI}_{OO}/\kappa^{OO}_{OI}$, $\kappa^{IO}_{OO}/\kappa^{OI}_{OO} = 2.5$; $\kappa^{IO}_{OI}/\kappa^{OI}_{OO} = 1$, and $\delta_1 = 0.075$, $\delta_2 = 0.06$, and $\delta_3 = 0.365$. $[I^+]_c$ is set equal to 0. The dotted line represents a fit of the constant-field equation to the results.

Modeling the results

The lack of a voltage-dependent effect of Na^+ on the amiloride-induced current inhibition implies that the channel occupancy by Na^+ cannot be a strong function of voltage, at least in the range of 0–200 mV. To explore whether this conclusion was reasonable, we examined a number of kinetic models based on a discrete state formalism (Eyring et al., 1949; Läuger, 1973; Hille, 1975; Andersen, 1989). The goal was to construct a minimal model, with the smallest possible number of energy barriers and wells, that could account for three general observations. First, there seems to be a common cation binding site for permeant ions, such as Na^+, impermeant ions, such as K^+, and high affinity blockers, such as amiloride. 15% of the voltage falls between the mucosal solution and this site. Second, the voltage dependence of the amiloride-induced block is the same at high and low [Na^+], which implies that channel occupancy by Na^+ is approximately voltage independent at both high and low [Na^+]. Third, the channels's current-voltage relationship in the presence of a large mucosal-to-cell [Na^+] concentration difference has a shape that is close to that predicted by the constant-field equation at small (negative) voltages, but is somewhat less steep at large positive voltages, and is not strongly dependent on the mucosal [Na^+], see Fig. 3 and Palmer (1984*a*).

Three general models were considered, one-site models, and two-site models. One-site models can be represented as

$$I_m^+ + C \underset{k_{Om}^I}{\overset{k_{Im}^O}{\rightleftharpoons}} IC \underset{k_{Ic}^O}{\overset{k_{Oc}^I}{\rightleftharpoons}} C + I_c^+.$$

Scheme 1

There is a single ion binding site, with 15% of the voltage falling between the mucosal solution and the site. Two versions of the model were examined: a one-site/sharp-barrier model that had sharp barriers on either sides of the site, such that the rate constants for transition across the barriers were simple exponential functions of potential; and a one-site/broad-barrier model, which was similar except that the inner barrier was broadened, such that rate constants for transitions across this barrier had a more complex voltage dependence (see below). The occupancy-voltage and current-voltage relations are expressed as

$$W(O) = (k_{Om}^I + k_{Oc}^I)/(k_{Om}^I + k_{Oc}^I + k_{Im}^O [I^+]_m + k_{Ic}^O [I^+]_c) \quad (6)$$

$$W(I) = (k_{Im}^O [I^+]_m + k_{Ic}^O [I^+]_c) / (k_{Om}^I + k_{Oc}^I + k_{Im}^O [I^+]_m + k_{Ic}^O [I^+]_c) \quad (7)$$

and

$$I(U) = N \cdot F \cdot \{k_{Im}^O [I^+]_m \cdot W(O) - k_{Om}^I \cdot W(I)\}, \quad (8)$$

where the Ws denote the probabilities of finding the channel empty or occupied, respectively, N denotes the number of channels and the ks are functions of voltage $k_{Im}^O = \kappa_{Im}^O \exp\{\delta_1 \cdot U\}$, $k_{Om}^I = \kappa_{Om}^I \exp\{-\delta_1 \cdot U\}$, $k_{Oc}^I = \kappa_{Oc}^I \exp\{-\delta_2 \cdot U - \omega \cdot U^2\}$, and $k_{Ic}^O = \kappa_{Ic}^O \exp\{-\delta_3 \cdot U - \omega \cdot U^2\}$. The ξs denote the rate constants at $V_T = 0$, the δs are the fractions of voltage that fall between the aqueous solutions, or the binding site, and the peak of the adjacent energy barrier, and ω is a "shape" parameter that denotes how rapidly the energy falls off from the peak ($\omega = [4 \cdot RT \cdot d] \cdot [\frac{d^2E}{dx^2}]^2$, where $E(x)$ is the potential energy, x is distance, and d denotes the channel length; see Andersen [1988] for further details). For a sharp barrier $\omega = 0$. The rate constants are subject to the constraints imposed by detailed balance: $\kappa_{Om}^I/\kappa_{Im}^O = \kappa_{Oc}^I/\xi_{Ic}^O$; and $2 \cdot \delta_1 + \delta_2 + \delta_3 = 1$.

Two-site models can be represented as

$$I_m^+ + C \underset{k_{IO}^{OO}}{\overset{k_{IO}^{OO}}{\rightleftharpoons}} IC \underset{k_{IO}^{OI}}{\overset{k_{OI}^{IO}}{\rightleftharpoons}} CI \underset{k_{OI}^{OO}}{\overset{k_{OO}^{OI}}{\rightleftharpoons}} C + I_c^+.$$

Scheme 2

The occupancy-voltage and current-voltage relations are expressed as

$$W(IO) = \{k_{IO}^{OO} [I]_m \cdot (k_{IO}^{OI} + k_{OO}^{OI}) + k_{OI}^{OO} [I]_c \cdot k_{IO}^{OI}\}/\text{Denom} \quad (9)$$

$$W(OI) = \{k_{OI}^{OO} [I]_c \cdot (k_{OI}^{IO} + k_{OO}^{IO}) + k_{IO}^{OO} [I]_m \cdot k_{OI}^{IO}\}/\text{Denom} \quad (10)$$

$$W(OO) = 1 - [W(IO) + W(OI)], \quad (11)$$

and

$$I(U) = N \cdot F \cdot [k_{OI}^{IO} \cdot W(IO) - k_{IO}^{OI} \cdot W(OI)], \quad (12)$$

where

$$\text{Denom} = k_{OI}^{IO} \cdot k_{OO}^{OI} + k_{IO}^{OI} \cdot k_{OO}^{IO} + k_{OO}^{IO} \cdot k_{OO}^{OI} + k_{IO}^{OO} [I]_m \cdot (k_{OI}^{IO} + k_{IO}^{OI} + k_{OO}^{OI}) + k_{OI}^{OO} [I]_c \cdot (k_{OI}^{IO} + k_{IO}^{OI} + k_{OO}^{IO}). \quad (13)$$

The ks are again functions of voltage: $k_{IO}^{OO} = \kappa_{IO}^{OO} \cdot \exp\{\delta_1 U\}$, $k_{OI}^{IO} = \kappa_{OI}^{IO} \cdot \exp\{\delta_2 \cdot U\}$, $k_{OO}^{OI} = \kappa_{OO}^{OI} \cdot \exp\{\delta_1 \cdot U\}$, $k_{OO}^{IO} = \kappa_{OO}^{IO} \cdot \exp\{-\delta_1 \cdot U\}$, $k_{IO}^{OI} = \kappa_{IO}^{OI} \cdot \exp\{-\delta_2 \cdot U\}$, $k_{OI}^{OO} = \kappa_{OI}^{OO} \exp\{-\delta_3 U\}$, where the ξs denote the rate constants at $U = 0$, and the δs denote the fractions of the voltage that affect the respective rate constants. The rate constants are subject to the constraint imposed by detailed balance: $\kappa_{IO}^{OO} \cdot \kappa_{OI}^{IO} \cdot \kappa_{OO}^{OI} = \kappa_{OI}^{OO} \cdot \kappa_{IO}^{OI} \cdot \kappa_{OO}^{IO}$; and $\delta_1 + \delta_2 + \delta_3 = 0.5$. For simplicity, the two-site models were restricted to the subset that had two binding sites 15% and 30% of the way through the channel and three symmetrical sharp energy barriers.

One-site models with sharp barriers could not account satisfactorily for the results. As shown in Fig. 4 (*dashed lines*) the occupancies predicted by these models decreased with increasing voltage especially in the range of +100 to +200 mV. In addition, the current-voltage relationships of these models were too steep in this voltage range, especially at high ion concentrations. (If the inner barrier was made highly asymmetric, the model would fit the results somewhat better. But the fit would not be as good as with the other models.)

The one-site models could simulate the experimental data if the energy barrier facing the inner solution was broadened. In this case, ion occupancy could be approximately constant between 0 and 200 mV. The current-voltage relationships were fairly linear over this voltage range and the shapes were similar at all concentrations (Fig, 4, *solid lines*).

Almost identical results could be obtained with three-barrier, two-site models in which the barriers were assumed to be sharp (Fig. 5). Both the occupancy-voltage and current-voltage relationships are similar to those of the one-site/broad barrier model over the experimental

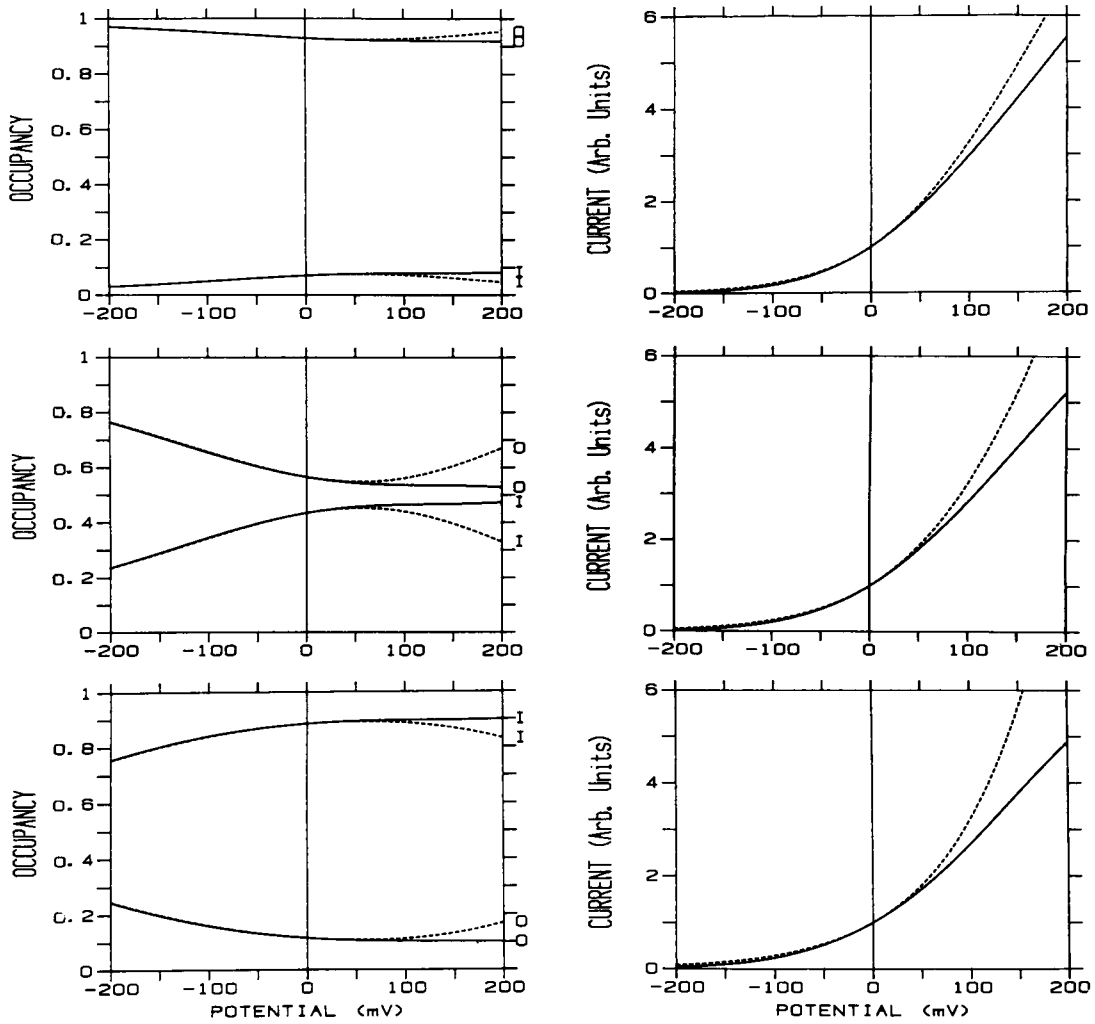

FIGURE 4 Occupancy-voltage (*left*) and current-voltage (*right*) relationships for a one-site-two-barrier model (see Scheme 1 and Eqs. 6–8). The parameters are: $\kappa^{I}_{Oc}/\kappa^{I}_{Om} = 0.3$ and $\delta_1 = 0.075$, $\delta_2 = 0.075$, $\delta_3 = 0.3$, and $\delta_4 = 0.55$. For simplicity, $[I^+]_c$ is set to 0. The three panels represent simulations for $\kappa^{O}_{Im}[I^+]_m/\kappa^{I}_{Om}$ equal to 0.1 (*top*), 1 (*middle*), and 10 (*bottom*), respectively. In each case, the dashed lines show the results when the inner barrier is sharp, and the solid lines show those when the inner barrier is broadened ($\rho = 0.225$). Ion occupancy increases as $[I^+]_m$ increases, but is in the broad-barrier model essentially independent of voltage over the experimental range: 0–200 mV. At 200 mV, the currents predicted by the sharp-barrier model are 1.2-fold (*top*), 1.5-fold (*middle*), and 2.0-fold (*bottom*) larger than those predicted by the broad-barrier model.

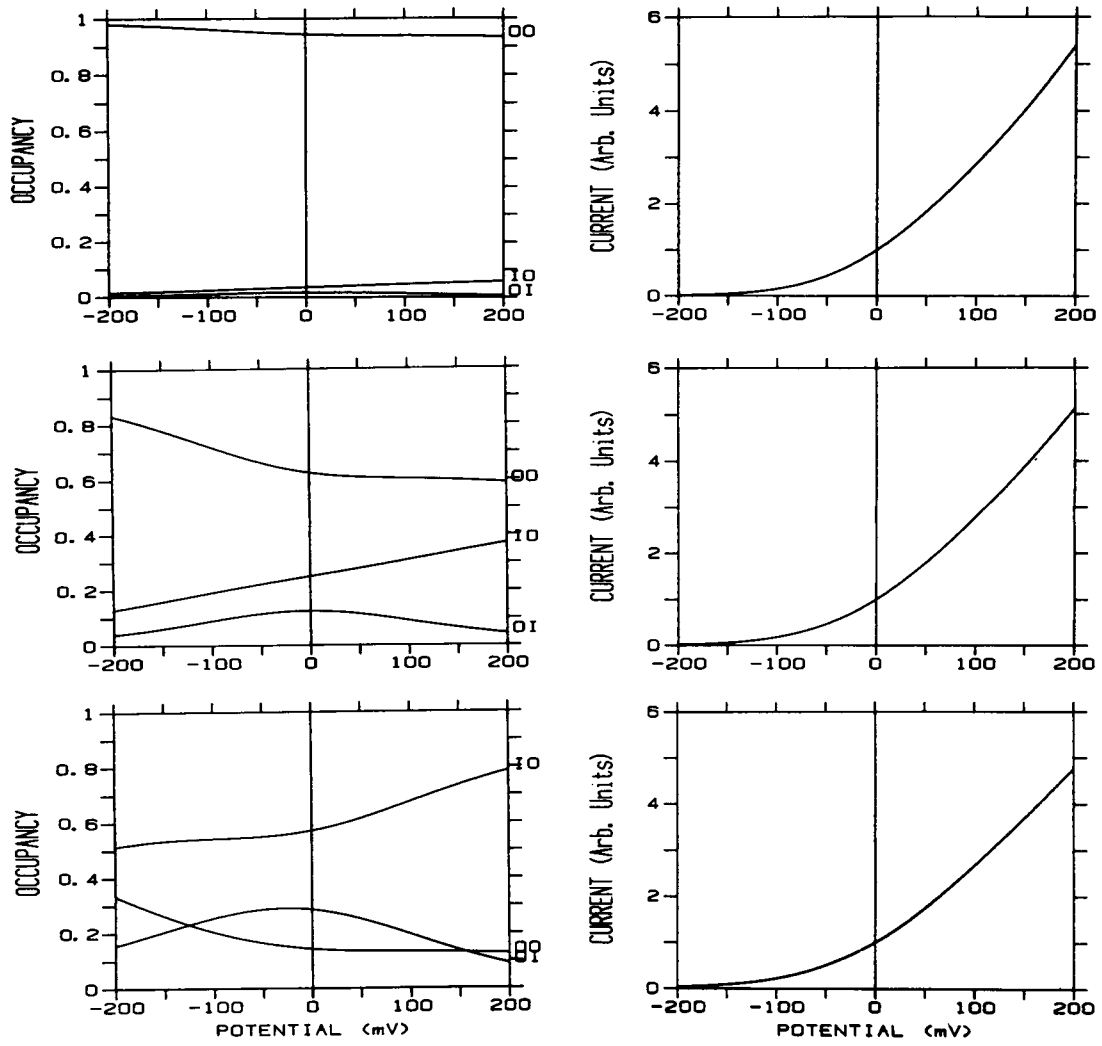

FIGURE 5 Occupancy-voltage (*left*) and current-voltage (*right*) relationships for a one-site-three-barrier model (see Scheme 2 and Eqs. 9–13). The parameters are: $\kappa_{00}^{10}/\kappa_{10}^{00} = \kappa_{00}^{01}/\kappa_{01}^{00}$; $\kappa_{00}^{10}/\kappa_{00}^{01} = 2$; $\kappa_{00}^{10}/\kappa_{00}^{01} = 1$; and $\delta_1 = 0.075$, $\delta_2 = 0.075$, and $\delta_3 = 0.35$. For simplicity $[I^+]_c$ is set equal to 0. The three panels represent simulations for $2 \cdot \kappa_{10}^{00}[I^+]_m/\kappa_{00}^{01}$ equal to 0.1 (*top*), 1 (*middle*) and 10 (*bottom*), respectively. (The factor of 2 arises from having two binding sites.) Occupancies of the left and right sites, (I,O) and (O,I), depend in opposite ways on voltage, while the number of unoccupied channels (0,0) is essentially independent of voltage over the experimental range: 0–200 mV.

voltage range. Thus a two-site model, with sharp energy barriers, can also account for the experimental results but at the cost of introducing two additional free parameters.

DISCUSSION

In this study we provide further evidence that the site of interaction of Na^+ and K^+ with the amiloride binding site in the toad bladder is within the pore of the apical sodium channels. This is implied by the increase in the competitive strength of impermeant cations (K^+ and guanidinium) but not permeant cations (Na^+ and Li^+) as the voltage driving the ions from the mucosal solution into the channel is increased.

By themselves these findings do not necessitate that the amiloride binding site is within the channel, although such an interpretation is consistent with evidence presented here and elsewhere (Palmer, 1984*a*, 1985 Warncke and Lindemann, 1985). Another possibility is that amiloride binds at an allosteric effector site, thereby causing a voltage-dependent conformational change in the channel heat closes the pore. Under this interpretation, occupancy of the channel by Na^+ or K^+ would

prevent the pore from closing. While we cannot exclude this possibility, we prefer the former interpretation, in part because the latter involves the postulation of an additional amiloride binding site as well as a voltage-dependent conformational change, which are not needed in the simple plugging model. In addition, the idea that the amiloride molecule itself senses a portion of the electric field across the membrane is supported by recent measurements of single channel kinetics by Marunaka and Eaton (1988). These authors found that the amiloride-induced inhibition of ion movement through sodium channels in the apical membrane of A6 cells had a voltage dependence similar to that observed with the toad bladder, but that the inhibition by 6-chloro-3,5-diaminopyrazine-2-carboxamide, an uncharged amiloride analogue, had no detectable voltage dependence. This suggests that the drug itself, rather than a gating mechanism within the channel, is sensing the transmembrane voltage.

It is in this context of interest to compare the block of epithelial sodium channels by amiloride with the closing of voltage-dependent sodium channels by tetradotoxin (TTX), which is also a guanidinium-based compound. The similarity between the two compounds was pointed out previously by Cuthbert (1976). In bilayer-incorporated batrachotoxin-modified voltage-dependent sodium channels, the inhibition by TTX is voltage-dependent (Krueger et al., 1983 Moczydlowski et al., 1984; Green et al., 1987), which suggests a fairly close analogy. Green et al. (1987) showed, however, that Zn^{2+}, which appears to block voltage-dependent channels by entering the pore, does not alter the voltage-dependence of the TTX-induced channel closures. This argues against a pore-plugging mechanism for this toxin's action on voltage-dependent sodium channels. It is thus likely that amiloride and TTX act in fundamentally different ways on their respective target channels.

The conclusion that Na^+ occupancy in the pore is not a strong function of voltage constrains how the translocation process should be modeled kinetically. Besides voltage-independent Na^+ occupancy, other features that were used to select a model were the presence of a binding site $\sim$ 15% of the way through the transmembrane electric field, which can be occupied by amiloride or by a variety of inorganic and organic cations (Palmer, 1984*a*, 1985) a current-voltage relationship that is flatter than would be the constant-field equation, particularly at large positive voltages and flux ratio exponents of close to 1 (Palmer, 1982*b*; Benos et al., 1983), which imply a lack of single filing. The goal was a minimal model, with the fewest barriers and wells, that could account reasonably well for these data.

One model that satisfies the demand for a voltage-independent occupancy by Na^+ is the two-site-three-barrier model (Fig. 5). This model could also be used to predict current-voltage relations similar to those observed, as well as the other channel features we have considered. It offers a plausible theoretical reconstruction of the experimental data.

The two-site model may not, however, be the minimal model compatible with the observed features. One-site models can also give satisfactory descriptions of the channels as long as at least one of the energy barriers is assumed to be broad (Fig. 4). The similarity in the conductive properties of channels with single sites and broad barriers and those with multiple sites and sharp barriers is discussed more generally elsewhere (Andersen, 1988).

We wish to emphasize that our goal in building kinetic models was not to construct a unique explanation of the data but to construct a plausible explanation. In fact, we believe we have constructed two plausible alternatives. We also believe that this exercise is instructive, in that it illustrates the difficulty in deciding on a unique kinetic model of channel behavior, particularly when the energy barriers are allowed to be broad, and the accessible concentration and voltage ranges are fairly limited.

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